Research Report

Laser Microdissection and Laser Pressure Catapulting for the Generation of Chromosome-Specific Paint Probes

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L. Schermelleh, S. Thalhammer, W. Heckl, H. Pösl[1], T. Cremer, K. Schütze[1] and M. Cremer

Ludwig Maximilians Universität, Munich, [1]Academic Hospital München-Harlaching, Munich, Germany

ABSTRACT

Chromosome-specific paint probes provide a powerful tool with wide applications in cytogenetic analysis. Here, we present a new approach using UV-laser microbeam microdissection in combination with laser-pressure catapulting, which allows the fast isolation of single chromosomes for the generation of chromosome-specific paint probes. To demonstrate the feasibility of this approach, single chromosomes were collected and amplified with degenerate oligonucleotide-primed PCR, hapten-labeled and hybridized onto normal metaphase spreads. Fluorescence in situ hybridization signals revealed specific painting of the respective chromosomes.

INTRODUCTION

Chromosome-specific DNA probes can be generated by isolating DNA from whole chromosomes or chromosomal subregions and its subsequent universal amplification by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR). Such paint probes allow the direct visualization of specific chromosomes or sub-chromosomal regions in metaphase spreads as well as chromosomal territories in interphase nuclei by fluorescence in situ hybridization (FISH). The availability of such paint probes has become an important tool for cytogenetic analysis with wide applications, ranging from research studies of chromosome territories to diagnostic use in clinical genetics (1,2).

Different methods are available for the collection of such samples. *(i)* Chromosomes isolated by flow sorting have been successfully applied for the generation of whole chromosome-specific libraries from various species, which usually originate from several hundreds of sorted chromosomes (11,16). Flow sorting works best on chromosomes that have a wide range of sizes or present discrete differences in base pair composition. *(ii)* The conventional microdissection approach uses fine glass needles for the collection of chromosomes (6,9,10) and also allows the isolation of subchromosomal regions of GTG (G-bands by trypsin using Giemsa)-banded chromosomes with high precision (7). However, needle dissection is tedious and needs especially skilled personnel. Furthermore, this method requires mechanical contact and therefore is sensitive to contamination.

In this paper, we present a method for microdissection and rapid collection of chromosomes that is solely based on laser micromanipulation. We demonstrate that this approach allows the fast generation of chromosome-specific paint probes originating from a few or only one copy of a chromosome without mechanical contact. The entire approach comprises *(i)* the mounting of metaphase spreads on ultrathin membranes, *(ii)* the optional destruction of unwanted genetic material adjacent to the target chromosome by laser ablation, *(iii)* the isolation of the separated chromosomes by laser dissection of the membrane around the target chromosome, *(iv)* the collection of the chromosome-membrane stack by laser-pressure catapulting (LPC) onto a collection device and *(v)* universal DNA amplification of the collected chromosomes by DOP-PCR and labeling of the amplified probe.

The chromosomal specificity of the obtained probes was demonstrated by hybridizing the labeled probes to normal metaphases.

MATERIALS AND METHODS

Materials

Metaphase chromosomes were obtained from cell cultures of a diploid kidney cell line from Chinese hamster (kindly provided by S. Müller, University of Munich, Germany) and from short-term cultures of human lymphocytes according to standard procedures.

Metaphase Chromosome Spreads

A piece of a 2 × 3-cm-sized, 1.35-µm-thick polyethylene naphthalate (PEN) or polyester-based (NYLA) membrane (provided by P.A.L.M. GmbH, Bernried, Germany) was placed on a pre-cleaned slide, fixed with Scotch® tape and UV-irradiated (254 nm) for 30 min. Mounting was facilitated by a drop of pure ethanol placed on the coverslip. Metaphase chromosome suspensions from the Chinese hamster cells or human lymphocytes fixed in 3:1 methanol:acetic acid were dropped onto the membrane. After 30 s, the slide was dipped briefly into 70% acetic acid to obtain well-spread metaphases, then dehydrated through an alcohol series (70%, 90% and 100%) and air-dried. Giemsa staining was performed in a 0.1% Giemsa solution for 10 min, slides were thoroughly rinsed with sterile, filtered water and air-dried. For GTG-banding, slides were aged at 56°C overnight and incubated in 0.1% trypsin for 20–40 s before Giemsa-staining.

Laser Microbeam Microdissection (LMM)

A commercially available UV-Laser Microbeam System (ROBOT-MICROBEAM; P.A.L.M. GmbH) was used as previously described (12). This system consists of a 337-nm nitrogen laser that is coupled into the light path of an inverted microscope and focused through an oil immersion objective (63× or 100× magnification, respectively) with high numerical aperture to yield a spot size of less than 1 µm in diameter (13). The microscope stage (Robot-Stage), the micromanipulator (Robot-Manipulator) and the laser micromanipulation procedure are computer-controlled. The microscope image is transferred by a video camera and displayed on a video screen. An energy of 0.2–0.3 µJ/pulse was used for ablation and of 0.5–0.6 µJ/pulse for membrane dissection.

LPC

For catapulting, the laser was focused slightly below the membrane. The isolated chromosome-membrane islets were ejected from the object slide with a single laser shot of 1–2 µJ/pulse and catapulted onto a collection device, held at a distance of <1 mm above the slide by a micromanipulator as shown in Figure 1, A and B. The collecting device consists of a small (1.5 × 3 mm) piece of glass heat-sealed to a pipet tip. Pure glycerol (0.1 µL) was placed on the collection glass to improve the adherence of the catapulted specimen and to facilitate their release into a reaction tube. Successful collecting of the catapulted membranes into the glycerol drop was monitored by focusing an objective of low magnification onto the collecting glass.

DOP-PCR

After LPC, the glass particle was transferred into a reaction tube and centrifuged at 1000× *g* for 3 min to get the glycerol drop with the selected chromosomes into the bottom of the tube. The glass piece was then removed using a clean forceps. The amplification of the sample DNA was performed with the partially degenerate universal primer 6MW (5′-CCG ACT CGA GNN NNN NAT GTG G-3′) (15) in a final volume of 25 µL of a PCR mixture containing 60 mM Tris-HCl, pH 8.5, 15 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 0.05% W-1-detergent (Life Technologies, Paisley, Scotland, UK), 200 µM of each dNTP, 1.5 µM 6MW primer and 1.5 U *Taq* DNA Polymerase (Life Technologies). Primary amplification was performed on a PROGENE Thermal Cycler (Techne, Princeton, NJ, USA) with the following cycling parameters: initial denaturation at 96°C for 3 min, 8 low-stringency cycles of 96°C for 1 min, 30°C for 1 min, 3-min transition of 30° to 72°C and 72°C for 2 min, followed by 35 high-stringency cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min and a final extension of 5 min at 72°C.

Two microliters of the primary amplification product were labeled with digoxigenin or biotin in a secondary-

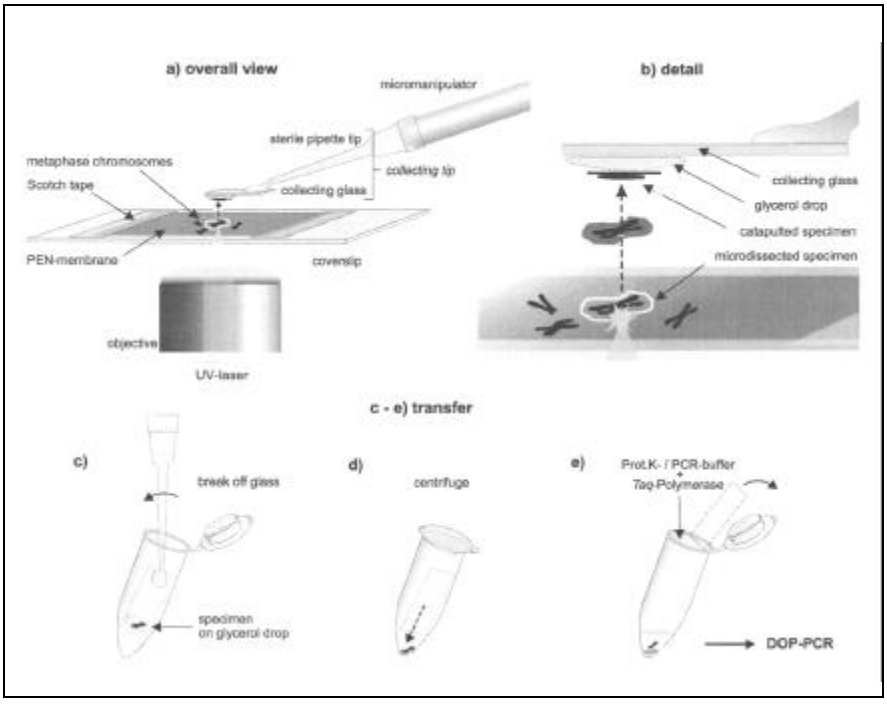

Figure 1. Schematic diagram of the LMM/LPC procedure.

label PCR in a volume of 50 µL of a PCR mixture containing 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, 50 mM KCl, 200 µM of dATP, dCTP and dGTP each, 160 µM dTTP, 40 µM Digoxigenin-11-dUTP or Biotin-16-dUTP (Boehringer Mannheim GmbH, Mannheim, Germany), 1.5 µM 6MW primer and 2 U *Taq* DNA polymerase. PCR conditions were as follows: initial denaturation at 94°C for 3 min, 20 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 30 s and final extension 72°C for 3 min. The probe size, checked on a 1% agarose gel, was in the range between 300 and 800 bp.

FISH

Approximately 50 ng/µL of the labeled probe with 1 µg/µL of the respective COT-1 DNA® (Life Technologies) were hybridized on metaphase chromosomes and detected according to standard procedures (8).

Photographs were taken with a Zeiss Axiophot® Epifluorescence Microscope (Carl Zeiss Jena GmbH, Jena, Germany). Digital images were obtained using a cooled charge-coupled device (CCD) camera (Photometrics, Tucson, AZ, USA) coupled to the microscope. Camera control and digital image acquisition were performed on a Cytovision system (Applied Imaging, Santa Clara, CA, USA).

RESULTS AND DISCUSSION

The LMM technique and the LPC procedure were recently described for the capture of single cells (14); however, to our knowledge, they have not yet been used for the collection of chromosomes and the generation of chromosome-specific paint probes. The LMM/LPC technique utilizes the light-pressure force caused by the high photon density within the narrow focus of the laser beam, and it differs significantly from the "cookie-cutter" method (4) and from the recently described laser-capture-microdissection (3), where an infrared heat-generating laser is used to melt the area-of-interest onto a transfer membrane. Figure 1 schematically outlines the principle of our approach.

The morphology and Giemsa staining of the metaphase chromosomes, spread on a UV-irradiated ultrathin supporting membrane, was comparable to those spread directly on glass slides (Figure 2, A and E). The laser microbeam was used to first cut the membrane around a chromosome-of-interest, yielding an islet with a diameter of 5–10 µm (Figure 2, B and G). Dissection of the membrane could be achieved using pulse energies of 0.5–0.6 µJ/pulse. Subsequently, the dissected membranes were catapulted right away onto the collection device by slightly focusing the laser below the membrane level (Figure 2, C and H). Using an energy of 1–2 µJ/pulse, the majority of catapulted membrane pieces could be recovered on the collection device (Figure 2D). If numerous copies of a homologous chromosome were isolated, they were all catapulted on one collecting device.

The chromosomal material could then be transferred on the collection device to a reaction tube and released into the tube by centrifugation.

For a first evaluation of our approach, we selected well-spread chromosomes as shown for a metaphase of the Chinese hamster in Figure 2, A–D. We wanted to test whether it is possible to isolate a single chromosome by the noncontact LMM/LPC procedure without destroying the genetic information. After optimizing the buffer conditions in the PCR, the DNA of the captured chromosomes was sufficiently accessible to serve as target DNA for a universal amplification. In case of one single copy, the amount of DNA for amplification was less than one picogram. Reverse-painting of the DOP-labeled probe on respective metaphase spreads resulted in the specific hybridization over the entire length of the captured chromosomes as shown for Chinese hamster chromosomes 1 (Figure 3A) and Y (Figure 3C), which shows a specific cross-hybridization of the homologous region on Xq. Thus, our results demonstrate that the laser energy used for membrane dissection, in a distance of 2–5 µm around the target chromosome and for the catapulting procedure, does not alter the target DNA, at least not to an extent that noticeably interferes with DOP-PCR amplification. This is probably due to the fact that the applied laser wavelength of 337 nm is sufficiently distant from the absorption maximum of DNA of 260 nm and that the effective laser energy is limited within a narrow focal area (5). As one copy of a chromosome is sufficient for the generation of a specific paint probe, the identification of individual chromosomes (e.g., by GTG banding before microdissection) is not absolutely necessary. This makes our approach espe-

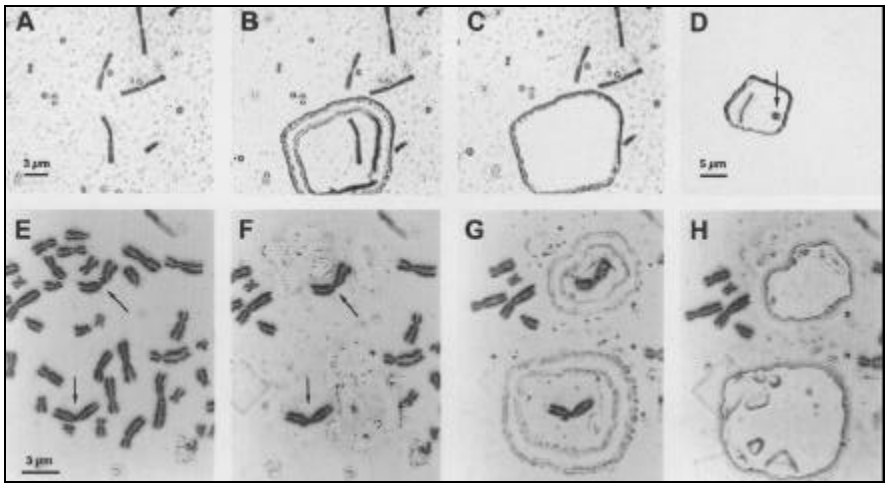

Figure 2. Isolation of metaphase chromosomes by LMM/LPC. Panels A–D show a Chinese hamster metaphase; Panels E–H show a human metaphase. (A) Chromosomes fixed on a PEN membrane; (B) isolation of hamster chromosome 1 by membrane dissection; (C) isolated region after catapulting; (D) monitoring of the catapulted chromosome-membrane stack on the collecting glass; arrow indicates spot caused by laser shot (16× magnification); (E) human metaphase chromosomes fixed on a polyester membrane; arrows indicate chromosomes 1; (F) ablation of numerous chromosomes around the chromosomes 1; (G) isolation of selected chromosomes 1 by membrane dissection; (H) isolated regions after catapulting.

cially attractive for chromosome-specific probe generation of species where unequivocal identification of specific chromosomes by size or banding analysis is difficult or even impossible. In such cases, to avoid the excess collection of identical chromosomes from different metaphases, the preparation of widely spread metaphases that allow the isolation of numerous chromosomes from one metaphase is highly desirable. Irrespective of this application, the identification of chromosomes by GTG banding wherever possible would be helpful for many purposes. Therefore, we have tested several conditions of banding procedures. However, the banding quality of membrane-mounted chromosomes was always less compared to chromosomes directly spread on glass slides and did not allow the reliable identification of all chromosomes from a membrane-mounted metaphase (data not shown).

A key feature for future applications of the LMM/LPC technique would be the ability to capture discrete subregions of a chromosome as, i.e., chromosome arms or finer sub-chromosomal regions. In principle, two approaches can be pursued for the isolation of such sub-chromosomal fragments. *(i)* A sub-chromosomal fragment could be directly isolated by cutting the underlying membrane along the respective target fragment. However, the dissection of membranes that are presently available requires a laser energy of at least 0.5 µJ/pulse, resulting in a cutting width of approximately 3 µm, including the structurally altered rim as shown in Figure 2B. Such cutting lines are not sufficiently fine for the precise isolation of an intact chromosomal subfragment. This present limitation could be overcome as soon as membranes become available that require less energy for cutting and thus will allow very fine cutting lines. *(ii)* Alternatively, the isolation of chromosomal subregions could be achieved by preceding ablation of unwanted genetic material adjacent to the target chromosomal fragment before dissecting the membrane

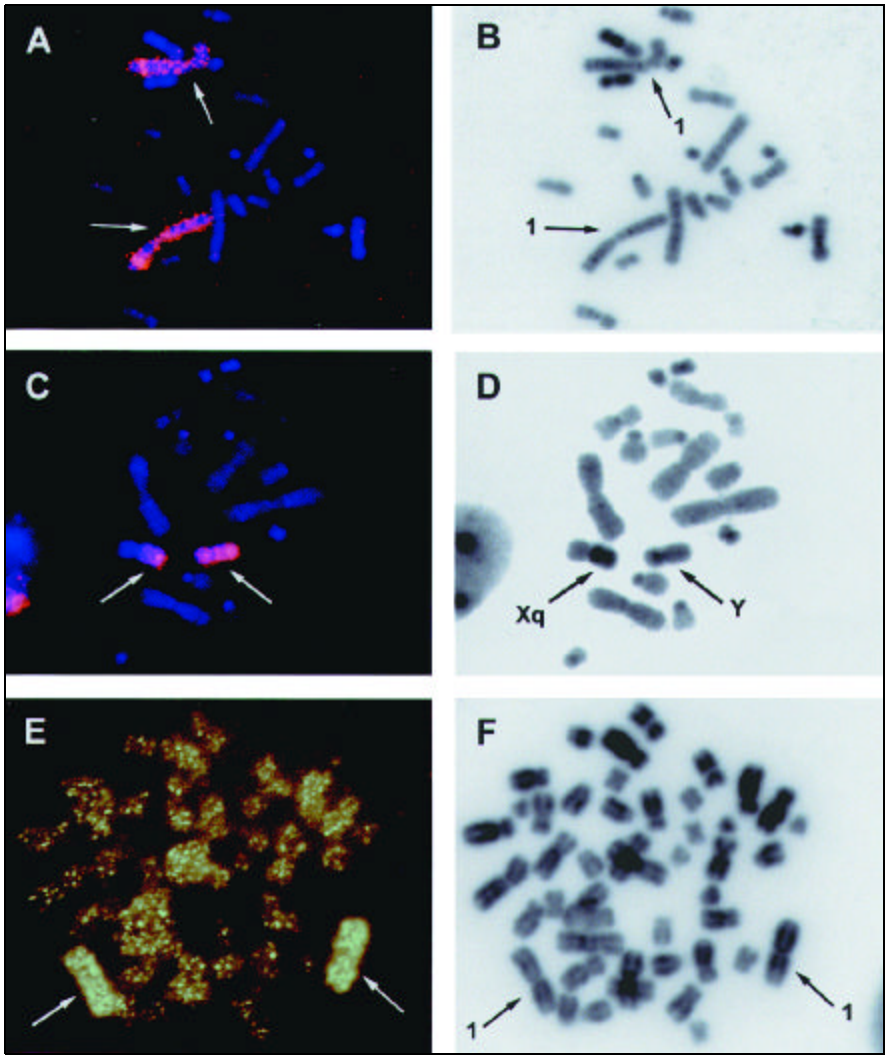

Figure 3. Hybridization of DNA probes obtained by LMM/LPC. (A) Hamster chromosome 1 probe derived from one isolated copy, detected with anti-DIG-Cy™3 and counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI); (B) Inverted DAPI picture of the same metaphase; (C) Hamster chromosome Y probe derived from one isolated copy of a GTG-banded preparation, detected with anti-DIG-Cy3 and counterstained with DAPI; note the specific cross-hybridization of the homologous region on Xq; (D) inverted DAPI picture of the same metaphase; (E) human chromosome 1 probe derived from 15 isolated copies detected with avidin-fluorescein isothiocyanate (FITC); some unspecific hybridization on other chromosomes is observed; (F) inverted DAPI picture of the same metaphase.

at a wider distance. This approach could also be used for the isolation of chromosomes where well-spread metaphases could not be achieved. Figure 2F shows an example of preceding laser-ablation of the chromosomes around the chromosomes 1 of a human metaphase spread.

What is questionable is whether the laser-ablation procedure destroys the ablated chromosomal material, not only structurally, but to an extent that even prevents any amplification by DOP-PCR.

In our experiments, we used an energy of 0.2–0.3 μJ/pulse for ablation of chromosomes. As shown in Figure 2F, this energy resulted in an optical destruction of ablated DNA but still left the underlying membrane intact and did not alter the shape of the chromosomes to be isolated. DOP-PCR resulted in a specific paint of the respective chromosome, as shown in Figure 3E for chromosome 1, where 15 copies had been isolated after ablation of the closely adjacent chromosomes of the respective metaphases. Thus, ablation of DNA material around the target DNA within a distance of down to 1 μm did not noticeably impair DOP amplification. However, in addition to the specific hybridization pattern, some unspecific hybridization signals on most of the chromosomes could be seen. Such an unspecific hybridization pattern was not observed using probes from sufficiently spread chromosomes, where preceding-ablation was not necessary. We assume that this unspecific hybridization is most probably due to an incomplete destruction of the ablated DNA, which, although invisible on the membrane after ablation, could still be DOP-amplified to some extent. This assumption is supported by our finding that DOP-PCR products of a catapulted membrane piece from which an entire metaphase had been visibly ablated, also resulted in some background hybridization. Contamination problems could be excluded, as amplifications from empty catapulted membrane pieces collected from the same slide did not result in any positive hybridization (data not shown).

We are currently working on improved conditions for the generation of background-free paint probes from sub-chromosomal regions. Once these conditions have been established, the combination of LMM and LPC will become a valuable method also for the generation of specific paint probes from sub-chromosomal regions.

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Address correspondence to:

Dr. Marion Cremer
Institute of Anthropology and Human Genetics
Ludwig Maximilians Universität
Richard-Wagnerstre 10/1
D-80333 Munich, Germany
Internet: marion.cremer@lrz.uni-muenchen.de